

Protective Effects of Ginkgo Biloba Dropping Pills Against Liver Ischemia/reperfusion Injury in Mice

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Research

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Abstract

Background: Liver ischemia-reperfusion (I/R) injury is an inevitable pathological phenomenon in various clinical conditions, such as liver transplantation, resection surgery, or shock, which is the major cause of morbidity and mortality after operation. *Ginkgo Biloba* Dropping Pill (GBDP) is a unique Chinese *Ginkgo Biloba* leaf extract preparation that exhibits a variety of beneficial biological activities. The aim of this study is to investigate the protective effects of GBDP on the liver I/R injury both *in vitro* and *in vivo*.

Methods: Hypoxia/reoxygenation (H/R) experiments were performed in AML-12 cells and primary hepatocytes, which were pretreated with GBDP (60 or 120 µg/mL) followed by incubation in a hypoxia chamber. Cell viability and cell apoptosis were detected by MTT assay and annexin V staining respectively. C57BL/6 mice were used to establish liver I/R injury model, and were pretreated with GBDP (100 or 200 mg/kg/day, i.g.) for two weeks. Liver damage was detected by plasma levels of alanine transaminase (ALT) and aspartate transaminase (AST). Liver necrosis and neutrophil infiltration were determined by H&E and myeloperoxidase immunohistochemistry staining. Finally, TUNEL staining and western blot analysis of apoptosis-related proteins were used to investigate the anti-apoptotic effect of GBDP.

Results: In the *in vitro* study, GBDP pretreatment improved the cell viability of AML-12 cells in H/R injury model. Similarly, the same result was found in the primary hepatocytes isolated from C57BL/6 mice. Moreover, GBDP decreased the number of apoptotic cells induced by H/R. In the *in vivo* study, oral administration of GBDP ameliorated liver injury evidenced by a significant decline in the levels of ALT and AST. Furthermore, the result of H&E staining showed that GBDP reduced the size of necrosis area. In addition, the decreased infiltration of neutrophils indicated that GBDP may play an anti-inflammatory effect. More importantly, GBDP reduced TUNEL-positive cells and the expression of Bax and caspase-3 in liver indicating GBDP has anti-apoptotic effects.

Conclusion: Our findings elucidated that GBDP has potential effects for protecting against liver I/R injury characterized by its anti-apoptotic, anti-necrotic, and anti-inflammatory properties, which would promisingly make a contribution to the exploration of therapeutic strategies in the liver I/R injury.

Background

Liver ischemia-reperfusion (I/R) injury is an inevitable pathological phenomenon in liver transplantation or liver resection, which is the major cause of morbidity and mortality after clinical liver transplantation [1]. Liver I/R injury leads to approximately 10% of early transplant failure and can result in a higher incidence of graft rejection [2]. Therefore, prevention and treatment of liver I/R injury should be addressed urgently in clinic. Despite extensive research, clinically effective interventions are still to be developed.

Liver I/R injury involves a biphasic process of ischemia-induced cell damage and reperfusion-induced inflammatory response [3]. When blood flow is interrupted, the cellular metabolism changes from aerobic to anaerobic due to the lack of oxygen supply, which can lead to various hepatocytes dysfunction [4].

Once the blood flows, various reactive oxygen species (ROS) are generated due to the reoxygenation of the ischemic liver tissue, which further aggravates the hepatocytes injury [5]. Liver I/R injury is characterized by hepatocyte damage, endothelial and kupffer cell swelling, neutrophil infiltration, vasoconstriction, ROS production, and platelet aggregation in sinusoids [1, 6]. Experimental evidence suggests that hepatocyte damage generally accompanies with hepatocellular necrosis and apoptosis, which are the major causes of hepatocytes death during liver I/R injury [7, 8]. Bax and caspase, important regulators of the intrinsic apoptosis, are involved in the initiation and execution of apoptosis, which is the key effect mechanism of liver I/R injury [9, 10]. In addition, inflammatory response is also a pathological mechanism of liver I/R injury. Kupffer cells are responsible for generation of proinflammatory factors, such as ROS and inflammatory cytokines to attract and activate neutrophils [4, 11, 12]. The infiltrated neutrophils further release ROS, inflammatory cytokines, and myeloperoxidase (MPO), all of which aggravate hepatocellular damage [1, 13]. Therefore, liver I/R injury is a series of events leading to necrosis, apoptosis, and hepatocytes dysfunction.

Ginkgo biloba L. is a traditional Chinese medicine commonly used to treat memory loss and improve blood circulation for thousands of years [14]. Recently, *Ginkgo biloba* extract (GBE) is one of the most extensively used herbal medicine in the world [15]. It is reported that GBE has a variety of beneficial biological activities, including antioxidation, anti-inflammation, anti-tumor, cardioprotective and neuroprotective effects [16–20]. A standardized *Ginkgo biloba* extract 761 (EGb 761) has been used as strong antioxidative and neuroprotective agent to treat neurodegenerative diseases [21]. Furthermore, several researches have indicated that EGb 761 protects against renal, myocardial, and cerebral ischemia-reperfusion injury [22–25], and improves hepatic DNA damage and liver microcirculation after warm ischemia [26, 27]. *Ginkgo biloba* Dropping Pill (GBDP) is a unique *Ginkgo biloba* leaf extract preparation produced in China for treating angina pectoris and cerebral infarction caused by blood stasis. A previous study reported that GBDP is different from EGb 761 in the content of components, quantitative analysis of the 21 different components that identified between EGb 761 and GBDP indicated that EGb 761 has more organic acids compared to GBDP, whereas GBDP has high levels of flavonols [28]. GBDP has been reported that it has antioxidative and neuroprotective effects in various conditions [29, 30]. Beyond that, the significant antagonistic effect on platelet-activating factor (PAF) receptor of GBE indicated that it may has beneficial effects on organs I/R injury. However, the effects of GBDP on liver I/R injury are still unclear. In this study, we investigated the effects of GBDP on hepatic I/R injury. The results showed that GBDP administration significantly inhibited liver injury by exhibiting anti-apoptotic, anti-necrotic, and anti-inflammatory effects both *in vitro* and *in vivo*. Taken together, as one of the most widely used Chinese medicine developed by modern science and technology worldwide, GBDP has a potential protective effect against liver I/R injury. Therefore, our study has a great significance to the application and development of GBDP, and provides a valuable strategy for the treatment of liver I/R injury that there are no effective therapeutic drugs currently.

Materials And Methods

Reagents

GBDP (batch number: A01J180506) were provided by Wanbangde Pharmaceutical Group Co., Ltd (Wenling, China). Dimethylsulfoxide (DMSO) and carboxymethyl cellulose sodium (CMC-Na) were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Primary antibodies of Bax (2772S), caspase-3 (9662S), and β -actin (4970S) were purchased from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Beyotime Biotechnology Co., Ltd (Shanghai, China).

Animals

Eight week-old male C57BL/6 mice weighing 22–25 g were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China) and maintained in a controlled environment (20–26 °C, 12-hour light/dark cycle) with *ad libitum* access to food and water. The animal experiments were approved by the Animal Care and Use Committee of Zhejiang University School of Medicine.

Isolation of primary hepatocytes and cell culture

Primary hepatocytes were isolated from C57BL/6 mice according to the method reported previously [4]. Briefly, primary hepatocytes were obtained by perfusion with 0.05% collagenase type IV (Sigma-Aldrich, St. Louis, MO). Then hepatocytes were filtrated through a 70 μ m cell strainer and were resuspended in Media mixed with 42% percoll (Sigma-Aldrich, St. Louis, MO) followed by centrifugation for 5 minutes at 1300 rpm. Hepatocytes were plated in six-well culture dish at a density of 1.5×10^6 cells/well and were cultured in Medium 199 (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA), 1% penicillin-streptomycin (Gibco, USA), 23 mM N-2-Hydroxyethylpiperazine-N-2-Ethane Sulfonic Acid (HEPES) (Sigma-Aldrich, St. Louis, MO), and 10 nM dexamethasone (Sigma-Aldrich, St. Louis, MO). The mouse hepatocyte cell line alpha mouse liver 12 (AML-12) cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco, USA) containing 10% FBS, 1% penicillin-streptomycin, 1% Insulin-Transferrin-Selenium-G Supplement (ITS) (Sigma-Aldrich, St. Louis, MO), and 40 ng/mL dexamethasone. Both AML-12 cells and primary hepatocytes in flasks were cultured in the cell incubator (Thermo Fisher, USA) at 37 °C with 5% CO₂.

Model of hypoxia/reoxygenation (H/R) injury

AML-12 cells or primary hepatocytes were incubated at 37 °C in a closed hypoxia chamber filled with N₂ in a tri-gas incubator (94% N₂, 5% CO₂, 1% O₂), followed by reoxygenation in normal culture conditions to establish the H/R model. Drug intervention with GBDP of two concentrations (60 or 120 μ g/mL) was performed 2 hours before the onset of hypoxia. The blank solution DMSO was served as control.

Model of liver I/R injury and drug treatment

The mice were divided into four groups at random: sham, I/R, I/R + GBDP (100 mg/kg), I/R + GBDP (200 mg/kg). Mice in I/R and I/R + GBDP groups went through 70% warm hepatic I/R injury as described

previously [31]. Briefly, the mice were placed supine for midline laparotomy after anesthetization to expose the liver. Murine hepatic artery and portal vein were isolated and clipped with microvascular clamp. After 45 minutes of ischemia, the clamp was removed. Blood or liver tissue samples were collected at 6 or 24 hours after reperfusion for subsequent experiments. Blood samples were centrifuged for 10 minutes at 4000 rpm, and the plasma was obtained for liver damage assessment. Mice in I/R + GBDP group were treated with GBDP in 1% CMC-Na by gavage once per day for two weeks, and 2 hours before surgery on the 15th day. The 1% CMC-Na solution was given to the other two groups served as control.

Cell viability assay

Cell viability was detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described [32]. AML-12 cells or primary hepatocytes were seeded into a 96-well plate at a density of 1×10^4 or 8×10^4 cells/well respectively. Cells were cultured in H/R conditions after 2-hour pretreatment of GBDP in different concentrations for 12 or 6 hours. Then, medium containing 0.5% MTT reagent was added to the each well. After incubation for 4 hours, the supernatants were removed and 100 μ L DMSO was added to dissolve the formed formazan crystals at room temperature for 10 minutes. The absorbance of the solution was measured at 580 nm using the Infinite M1000 Pro (TECAN, Germany).

Annexin V staining

AML-12 cells were cultured in hypoxia chamber for 12 hours, and then reoxygenated for 22 hours. Cells were stained with an Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit according to the manufacturer's instructions (BD Biosciences, San Jose, CA). Briefly, AML-12 cells seeded in 6-well plates were collected and resuspended in $1 \times$ binding buffer to the final concentration of 1×10^6 cells/mL. Then 100 μ L cell resuspension was incubated with 5 μ L Annexin V-FITC and 5 μ L PI for 15 minutes at room temperature in dark. Next, 400 μ L $1 \times$ binding buffer was added to end the incubation. Apoptotic rate was assayed by Accuri™ C6 flow cytometer (BD Bioscience, San Jose, CA) in 1 hour.

Cell lysis and Western blot analysis

Preparation of whole cell or tissue lysates and western blot analysis were performed as described previously [33]. Briefly, the cells or tissues were lysed in the lysis buffer, which was made from Tris-HCL, NaCl, EDTA, glycerol, Triton X-100, Nonidet P-40, dithiothreitol, and phenylmethylsulfonyl fluoride, supplemented with protease and phosphatase inhibitors (Roche Diagnostics, Germany). The lysates were centrifuged for 15 minutes at 10000 g, and the protein concentrations were quantified by Bradford method using Quick Start™ Bradford $1 \times$ Dye Reagent (Bio-Rad, Hercules, CA). Lysates with equal amount of six mice from the same group were mixed together to be one sample. Protein samples were electrophoresed by 9–12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) gels (10–20 μ g of protein/lane) and separated protein was transferred to polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Germany). The membranes were blocked in Tris-Buffered Saline with Tween (TBST) containing 5% skim milk for 1 hour at room temperature, which was followed by incubation with

primary antibodies overnight at 4 °C. After washing three times in TBST, the membranes were incubated with the corresponding HRP-conjugated secondary antibodies for 1 hour at room temperature. All primary antibodies were used at a 1:1000 dilution, and secondary antibodies were used at the dilution of 1:2000. Protein bands were visualized by using a ChemiDoc™ XRS + system (Bio-Rad, Hercules, CA) with chemiluminescence substrate reagents (Bio-Rad, Hercules, CA) and quantified by using ImageJ software.

Liver damage assessment

Plasma concentrations of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), indicators of hepatocellular injury, were assayed by an automated biochemical analyzer (Cobas C8000, Roche, USA) following the manufacturer's instructions.

Histological analysis

After fixation in 10% formaldehyde, liver sections were embedded in paraffin for hematoxylin and eosin (H&E), MPO immunohistochemical, and Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick and labeling (TUNEL) staining. The liver tissues were sectioned at 5 µm thickness and stained with H&E to visualize the pattern in necrotic areas of the liver. The infiltration of neutrophils was detected by MPO staining. Liver sections were incubated with MPO primary antibody (1:1000, Servicebio) at 37°C for 1 hour, followed by incubating with HRP-conjugated goat anti-mouse secondary antibody (1:200, Servicebio). TUNEL staining was performed to determine DNA fragmentation using an *In Situ* Cell Death Detection Kit (Roche Diagnostics, USA) according to the manufacturer's instructions as described previously [34]. Images were captured using a fluorescence microscope (Nikon Eclipse Ti-SR, Japan).

Statistical analysis

All statistical analyses were performed with GraphPad Prism 5.0, and the data was expressed as the mean ± standard deviation (SD). Statistical differences between two groups were calculated by two-tailed Student's *t*-test, and one-way ANOVA was used for comparisons among multiple groups followed by Dunnett's multiple comparison test. Statistical differences with *p* values less than 0.05 were considered significant.

Results

GBDP improved cell viability in the H/R-induced hepatocellular injury model

H/R, a commonly used model of ischemia/reperfusion injury *in vitro*, was performed to explore the extent of cellular damage [4]. AML-12 cell line and primary hepatocytes were used to investigate the protective effects of GBDP against H/R-induced hepatocellular injury. Flow diagram of *in vitro* experiment was shown as Fig. 1a. We first investigated the time point of optimal H/R injury in AML-12 cells and primary hepatocytes. Results showed that the cell injury of AML-12 cells induced by H/R was maximized after 24 hours of reperfusion (Fig. 1b), and the cell injury of primary hepatocytes was maximized after 3 hours of

reperfusion (Fig. 1c). Next, we constructed H/R injury model of AML-12 cells or primary hepatocytes at indicated time point. Pretreatment of GBDP (60 or 120 µg/mL) for 2 hours improved cell viability of AML-12 cells injured by H/R (Fig. 1d). Similarly, the same result can be found in primary hepatocytes (Fig. 1e). Altogether, these results indicated that GBDP has protective effects in hepatocellular injury induced by H/R.

GBDP pretreatment inhibited apoptosis of AML-12 cells induced by H/R

Apoptosis is an important indicator, which reflects the degree of liver injury. Apoptosis of AML-12 cells induced by H/R was increased as evidenced by experiment of Annexin V staining. As shown in Fig. 2a, H/R injury dramatically increased the number of annexin V-positive cells, while GBDP pretreatment dose-dependently decreased apoptosis of hepatocytes. The experiment was repeated at least three times and quantitative result of apoptotic percentage was shown in Fig. 2b. These results indicated that GBDP has an anti-apoptotic effect in H/R-induced injury

Oral administration of GBDP alleviated hepatic injury in mice

To explore the *in vivo* efficacy of GBDP, we constructed a model of liver I/R injury in mice. Flow diagram of *in vivo* experiment was shown in the Fig. 3a. Mice were treated with GBDP by gavage once per day for two weeks, and 2 hours prior to ischemia on the 15th day. The plasma levels of ALT and AST are sensitive indicators of acute liver injury, which go down after 24 hours [35]. Thus, the blood samples were collected after 6 hours of reperfusion. The levels of ALT and AST were significantly elevated after liver I/R injury, while GBDP administration decreased them in a dose-dependent manner (Fig. 3b and c). More importantly, the result of H&E staining showed that both low and high doses of GBDP significantly reduced the liver necrosis found widely in I/R group (Fig. 3d). These findings indicated that GBDP plays a potential role to protect against liver I/R injury.

GBDP reduced neutrophil infiltration in I/R-injured liver tissue

To further verify the protective role of GBDP on the neutrophil infiltration after liver I/R injury, MPO immunohistochemical staining were performed. MPO is an enzyme predominantly stored in neutrophil granules, which can be used to quantify neutrophil infiltration in the liver [4]. The results showed that the number of MPO-positive cells was significantly reduced by GBDP administration indicating that the hepatic infiltration of neutrophils was inhibited (Fig. 4a). Meanwhile, MPO H-Score was also declined in GBDP-treated groups (Fig. 4b). These results further confirmed the beneficial effects of GBDP on infiltration of inflammatory cells caused by I/R injury.

GBDP attenuated hepatocytes apoptosis induced by liver I/R injury in mice

In addition to liver necrosis and infiltration of inflammatory cells, hepatocellular apoptosis in liver has also been explored. Firstly, the number of apoptotic cells was determined by TUNEL staining. Both low and high doses of GBDP massively decreased the number of apoptotic cells markedly increased in I/R group (Fig. 5a). Next, proapoptotic proteins of Bax and caspase-3 were detected by western blotting. The mixture lysate sample of each group was prepared as described previously [34]. Consistent with the histological results, GBDP downregulated the protein expression of Bax and caspase-3 elevated by I/R injury (Fig. 5b). Experiments were performed at least three times and quantitative results of Bax and caspase-3 were shown in Fig. 5c and d. These results demonstrated that GBDP has a strong inhibitory effect on liver I/R injury-induced hepatocellular apoptosis in mice. Overall, GBDP has protective effects on the liver I/R injury-induced apoptosis, necrosis, and neutrophil infiltration. A brief diagram of this study was shown in Fig. 6.

Discussion

Liver transplantation is an effective therapeutic method for treatment of end-stage liver disease. However, there is a huge disparity between the number of liver organs available for transplantation and the number of patients waiting for the liver transplantation, leading to an increase in the mortality of patients on the waiting list [6]. Therefore, it is important to improve the success rate of liver transplantation for a small number of patients who have received donor liver. Liver I/R injury is the major underlying cause of graft non-function or late dysfunction after liver transplantation [36]. Hence, prevention and reduction of I/R injury is one of the key factors for successful liver transplantation.

As one of the most universally used herbal medicinal product [37], GBE has a wide range of pharmacological properties. Numerous studies have shown that GBE has the characters of inflammation reduction, free radical scavenging, anti-tumor and nervous system activity enhancement [19, 38, 39]. It is well known that the liver I/R injury generally accompanies with apoptosis, accumulation of pro-inflammatory cells, and ROS generation [3]. Moreover, one of the critical active components of GBE, ginkgolides have been found to exhibit an antagonistic effect on PAF receptor specifically and selectively, which is deemed to associate with the protective effect in I/R injury [40–42]. For this condition, GBE is expected to be developed as a candidate drug for treatment of hepatic I/R injury. Nowadays, there are various commercial *Ginkgo Biloba* extract preparations in the market, and GBDP is a unique and popular one produced in China. According to these studies, we hypothesized GBDP could be an alternative therapy for preventing liver I/R injury.

In this study, we first conducted hepatocytes H/R injury model *in vitro*, a commonly used tool in liver I/R research to mimic cellular damage in the pathological process of I/R [4]. A significant increase in cell viability was observed in AML-12 cells pretreated by GBDP after H/R stimulation. Primary hepatocytes

provide an acceptable reflection of the hepatic *in vivo* situation [43], the same result observed in primary hepatocytes further confirmed our conclusion that GBDP protected against hepatocytes H/R injury.

Apoptosis of hepatocytes is one of the most important types of cell death in the progression of hepatic I/R injury [44]. Hepatocyte dies through active suicide in response to the overwhelming cellular stress, which is termed “apoptosis” [45]. During this physiological process, the activation of caspases results in the cleavage of proteins, then activation of nucleases that cleave DNA into fragments subsequently, leading to cell death [46]. Cells with fragmented DNA could be identified as apoptotic cells by TUNEL assay, which is a common detection way of apoptosis [47]. Furthermore, apoptosis is controlled by multiple genes including caspase family. Overexpression of Bax, an apoptotic protein, could cause caspase-9 mediated programmed cell death and ultimately upregulate caspase-3, which is considered as a final step towards apoptosis [48]. In our results, flow cytometry data showed that the apoptosis induced by H/R injury was dose-dependently alleviated by GBDP pretreatment in AML-12 cells. Consistent with the results of the *in vitro* experiments, subsequent animal experiments confirmed that GBDP administration reduced apoptosis in I/R-injured liver tissues, which has been shown in downregulating the expression of pro-apoptotic proteins and reducing the number of TUNEL-positive cells. Similar to these results, GBE has been reported to reduce the protein levels of Bax and caspase-3 in liver fibrosis model, which further confirmed the hepatoprotective effect of GBE through the anti-apoptotic effect [49].

As one kind of sterile inflammation, liver I/R injury is marked by abundant infiltration of neutrophils [50]. Intravital microscopy was used in mice hepatic I/R injury model to show massive recruitment of neutrophils to the site of hepatic injury [51]. Accumulated Neutrophils in the hepatic parenchyma release ROS and proteases, which results in hepatocytes damage and hepatic sinusoids destruction [52]. Thereby, several studies emphasized the strategies of inhibiting neutrophils recruitment to reduce I/R-induced liver injury [53, 54]. Analysis of infiltrated neutrophils in liver has been mainly assessed by histologic sections or activated markers such as MPO [51]. MPO is a peroxidase enzyme and most abundantly expressed in neutrophils. It is stored in azurophilic granules of the neutrophils and release into the extracellular space upon neutrophils recruitment [4]. In some ways, the level of MPO represents the degree of neutrophils infiltration. Therefore, we performed the immunohistochemical staining of MPO to analyze the infiltration of neutrophils in our study. GBDP downregulated the neutrophils infiltration, which was consistent with the anti-inflammatory effect of GBE elucidated in previous studies [55]. Further experiments should be performed to study the protective mechanisms of GBDP on liver I/R injury by interfering with inflammatory responses.

Conclusion

In conclusion, the present study elucidated that GBDP exerted a protective function on liver I/R injury *in vitro* and *in vivo*, as indicated by reducing apoptosis, decreasing the release of plasma aminotransferases, and improving I/R associated histopathologic changes to play anti-apoptotic and anti-inflammatory effects respectively. Overall, our results demonstrate a beneficial function of GBDP that may expected to be a candidate therapeutic agent to prevent liver I/R injury.

Abbreviations

ALT, alanine aminotransferase; AML-12, alpha mouse liver 12; AST, aspartate aminotransferase; Bax, B cell lymphoma-2-associated X; CMC-Na, carboxymethyl cellulose sodium; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; EDTA, Ethylene Diamine Tetraacetic Acid; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GBDP, *Ginkgo Biloba* Dropping Pill; GBE, *Ginkgo Biloba* extract; H&E, hematoxylin and eosin; HEPES, N-2-Hydroxyethylpiperazine-N-2-Ethane Sulfonic Acid; H/R, hypoxia/reoxygenation; HRP, horseradish peroxidase; I/R, ischemia/reperfusion; ITS, insulin-Transferrin-Selenium-G Supplement; MPO, myeloperoxidase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PAF, platelet-activating factor; PAGE, polyacrylamide gel electrophoresis; PI, fluorescein isothiocyanate; PVDF, polyvinylidene difluoride; ROS, reactive oxygen species; SD, standard deviation; SDS, sodium dodecyl sulphate; TBST, Tris-Buffered Saline with Tween; TUNEL, Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick and labeling.

Declarations

Acknowledgements

Not applicable

Authors' contributions

X.F., X.L. and Z.W. designed the study. Z.W. and P.Z. performed the study and wrote the manuscript. X.F. and X.L. revised the manuscript. Q.W., X.S. and J.Z. provided GBDP and support. All authors read and approved the final manuscript.

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Availability of data and material

All data and materials in the current study are included in this published article.

Ethics approval and consent to participate

The animal experiments were approved by the Animal Care and Use Committee of Zhejiang University School of Medicine.

Consent for publication

Not applicable

Competing interests

The authors declare no competing interests.

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Figures

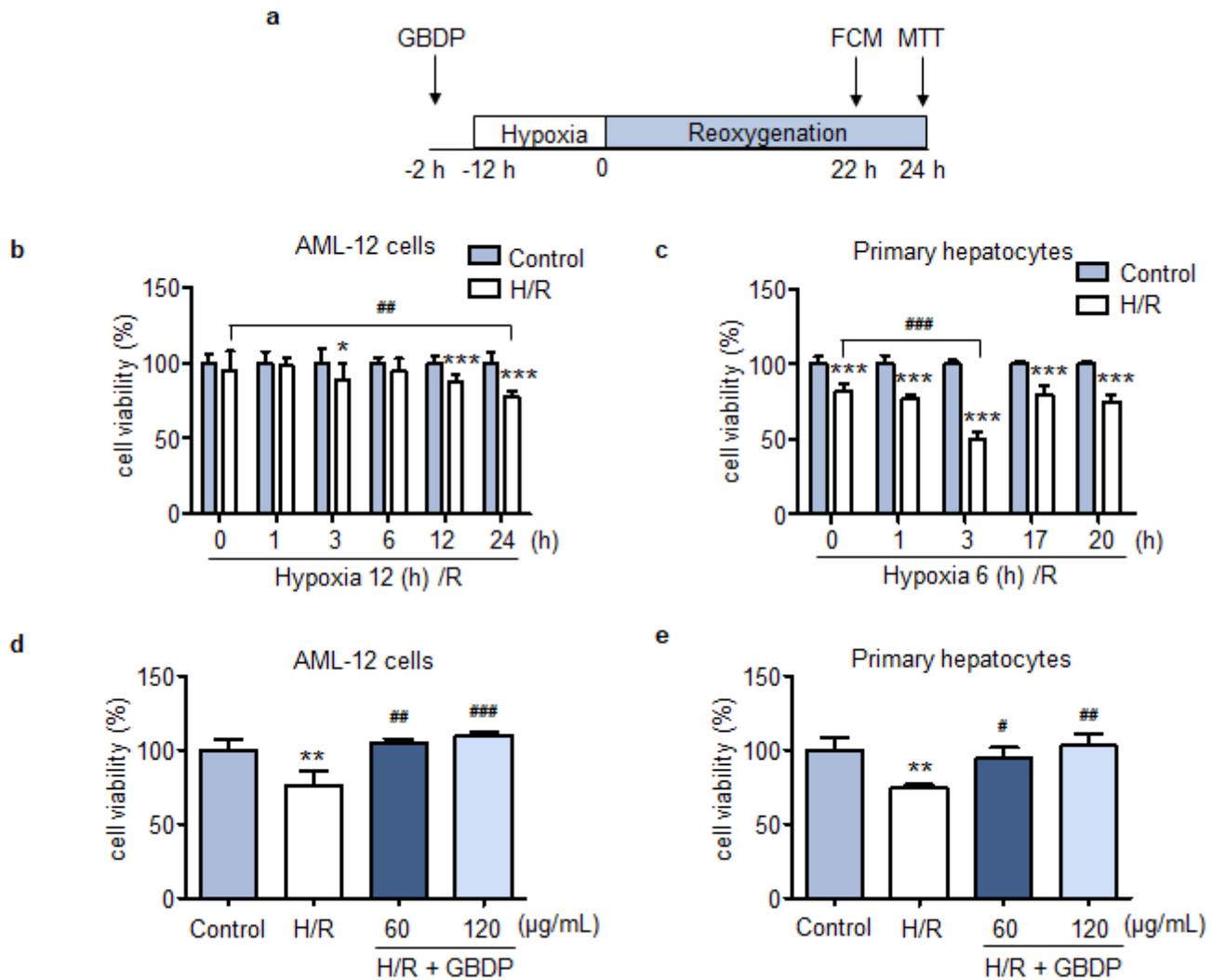


Figure 1

GBDP pretreatment improved cell viability injured by H/R. (a) Flow diagram of the in vitro experiment. (b) Construction of hepatocellular H/R injury model. AML-12 cells or (c) primary hepatocytes were cultured in hypoxia chamber for 12 or 6 hours and reoxygenated for various time periods. The cell viability was determined by MTT assay. (d) AML-12 cells treated with or without GBDP (60 or 120 $\mu\text{g}/\text{mL}$) were cultured in the hypoxia chamber for 12 hours and reoxygenated for 24 hours. Cell viability was determined ($n = 3$ per group). (e) Primary hepatocytes were exposed to 6-hour hypoxia and 3-hour reoxygenation after pretreatment of GBDP, and cell viability was detected ($n = 3$ per group). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ versus control group; # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ versus H/R group.

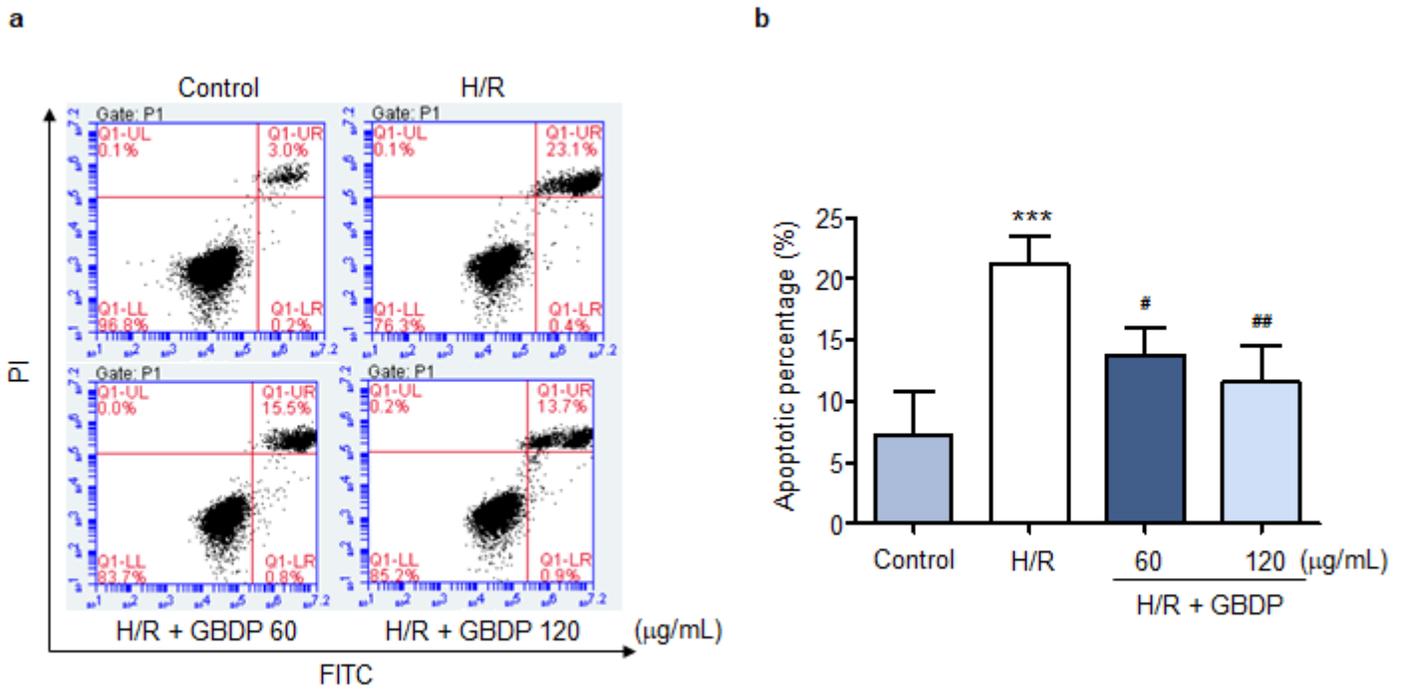


Figure 2

Apoptosis of AML-12 cells induced by H/R injury was inhibited by GBDP pretreatment. (a) Apoptosis was determined by Annexin V staining. AML-12 cells were pretreated with or without GBDP (60 or 120 $\mu\text{g}/\text{mL}$) for 2 hours. Cells were cultured in the hypoxia chamber for 12 hours and reoxygenated for 22 hours. (b) Apoptotic rate of AML-12 cells was shown on the right (n = 3 per group). ***p < 0.001 versus control group; #p < 0.05, ##p < 0.01 versus H/R group.

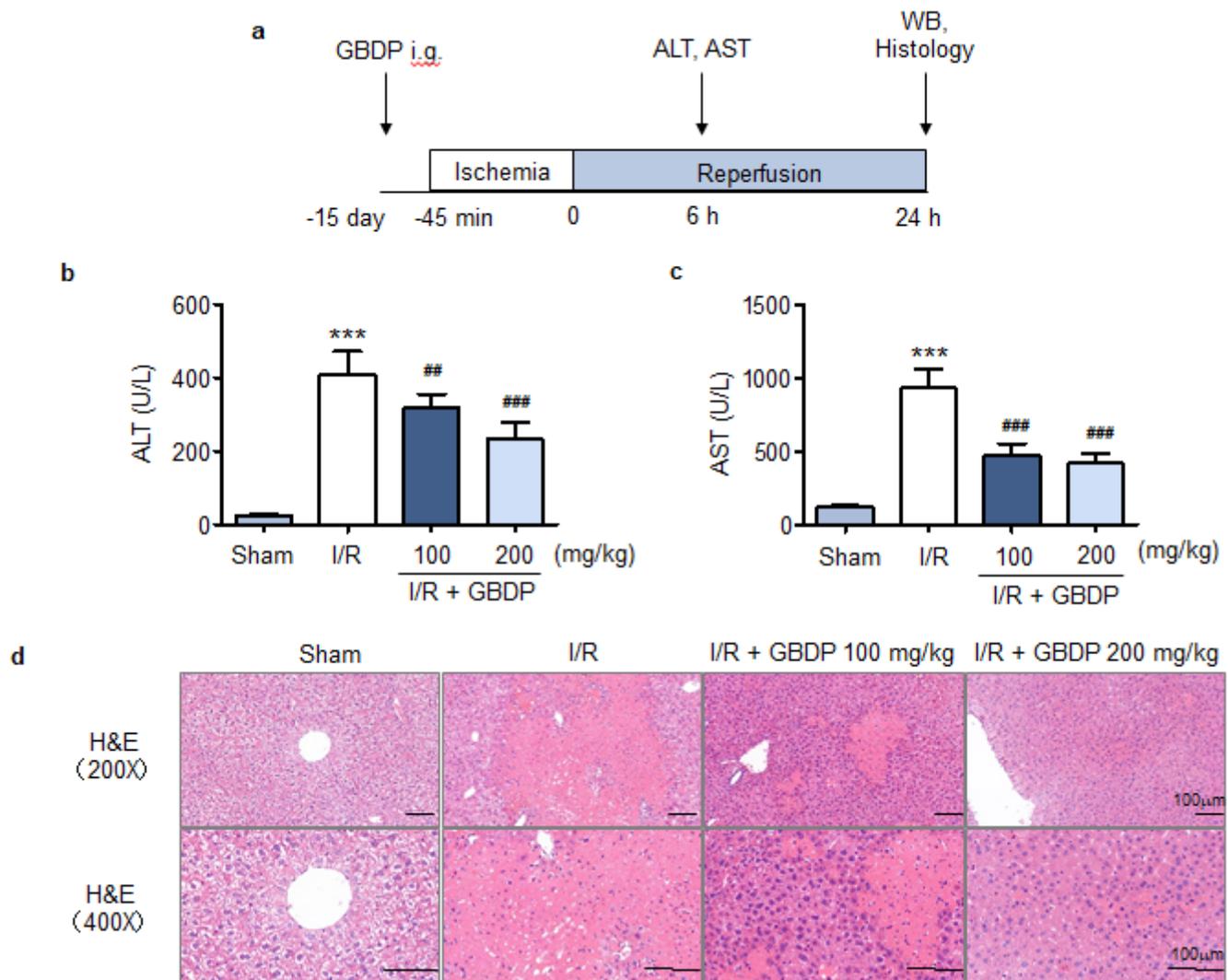


Figure 3

GBDP pretreatment attenuated the liver I/R injury in mice. (a) Flow diagram of the in vivo experiment. Samples of blood and liver tissue were collected, and the following analyses were performed: ALT and AST (6 hours of reperfusion), western blotting for apoptotic proteins and histology (24 hours of reperfusion). (b) The plasma levels of ALT and (c) AST were analyzed after 6 hours of reperfusion. Values were expressed as mean \pm standard error of the mean (n = 6 per group). (d) After 24 hours of reperfusion, liver necrosis was assessed by H&E staining. Bars = 100 μ m. ***p < 0.001 versus Sham group; ##p < 0.01 and ###p < 0.001 versus I/R group.

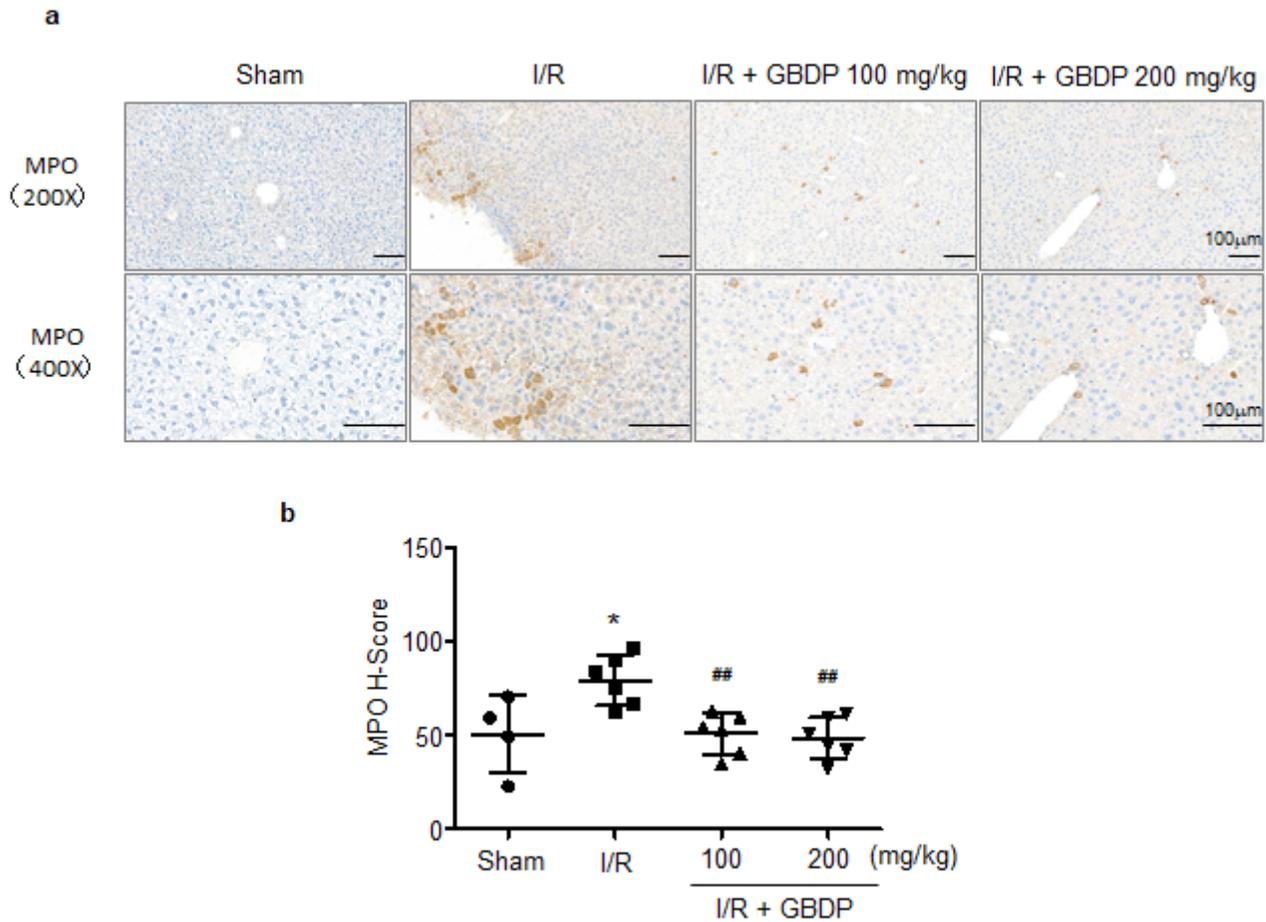


Figure 4

GBDP administration attenuated neutrophil infiltration in I/R-injured liver. (a) After 24 hours of reperfusion, neutrophil infiltration was assessed by MPO immunohistochemical staining. Bars = 100 μ m. (b) Histochemistry score of MPO was shown (n = 4-6 per group). *p < 0.05 versus Sham group; ##p < 0.01 versus I/R group.

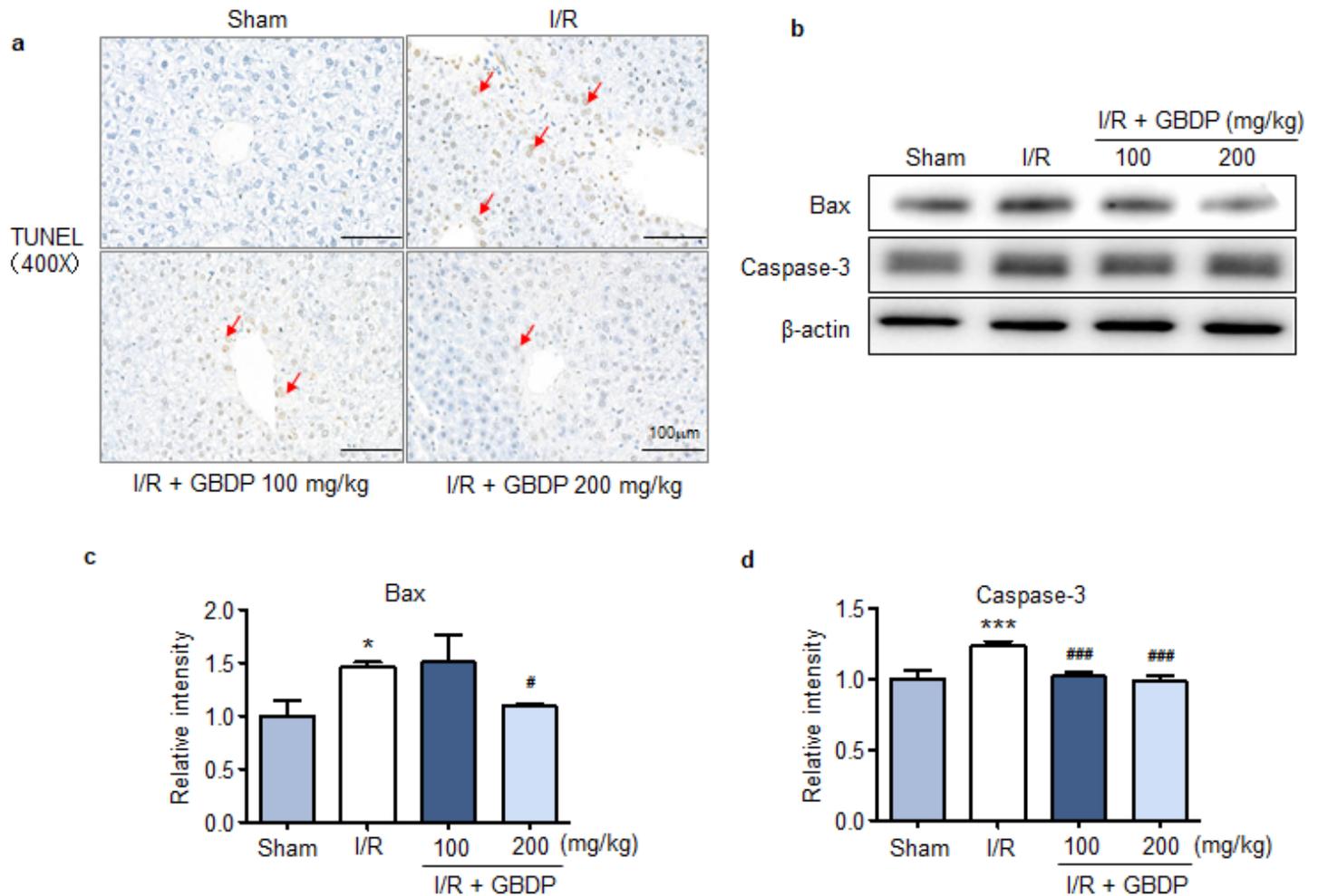


Figure 5

Liver I/R injury-induced hepatocellular apoptosis was inhibited by GBDP pretreatment in mice. (a) Apoptotic cells were detected by TUNEL staining after 24 hours of reperfusion. Bars = 100 μ m. (b) Apoptotic protein expression of Bax and caspase-3 in reperused liver tissue was analyzed by western blotting. Lysate sample of each group was a mixture of six mice from the same group (n = 6 per group). β -actin was used as a loading control. The mixture sample of each group was prepared three times and the experiments were repeated at least three times. (c) The quantification results of Bax and (d) caspase-3 were shown. * p < 0.05 and *** p < 0.001 versus Sham group; # p < 0.05 and ### p < 0.001 versus I/R group.

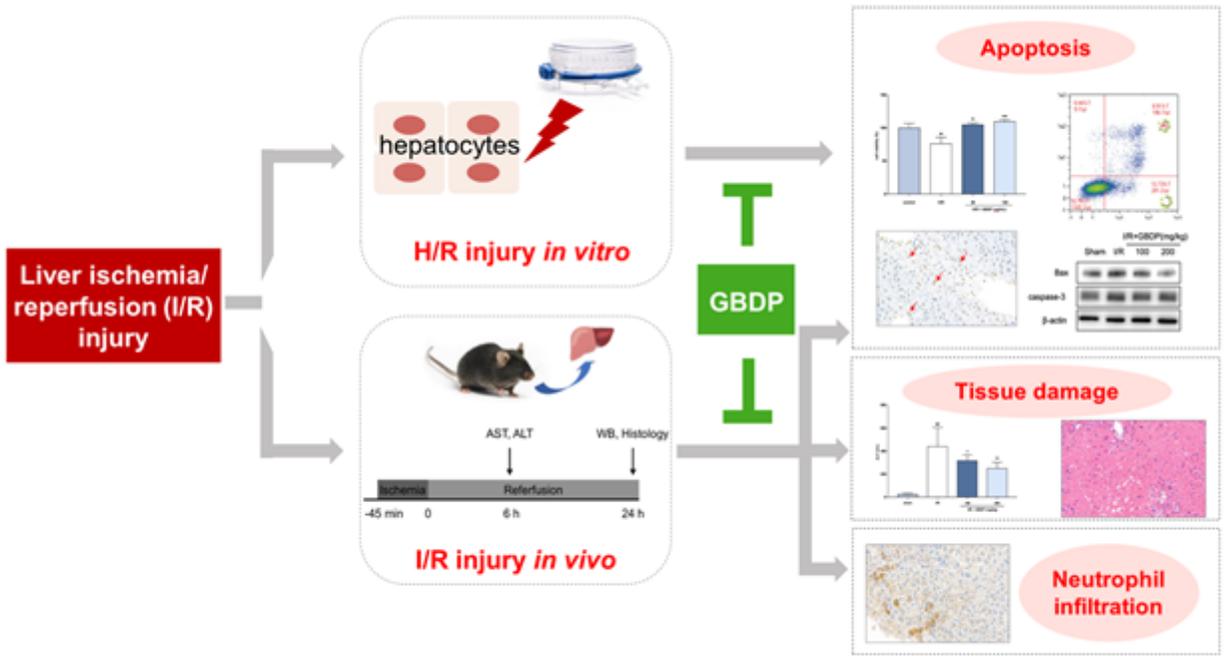


Figure 6

A brief diagram of this study was shown. GBDP significantly inhibited H/R-induced hepatocytes injury in vitro and I/R-induced liver injury in vivo.